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Bacteriophage-Mediated Control of a Two-Species Biofilm Formed by Microorganisms Causing Catheter-Associated Urinary Tract Infections in an *In Vitro* Urinary Catheter Model

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Microorganisms from a patient or their environment may colonize indwelling urinary catheters, forming biofilm communities on catheter surfaces and increasing patient morbidity and mortality. This study investigated the effect of pretreating hydrogel-coated silicone catheters with mixtures of *Pseudomonas aeruginosa* and *Proteus mirabilis* bacteriophages on the development of single- and two-species biofilms in a multiday continuous-flow *in vitro* model using artificial urine. Novel phages were purified from sewage, characterized, and screened for their abilities to reduce biofilm development by clinical isolates of their respective hosts. Our screening data showed that artificial urine medium (AUM) is a valid substitute for human urine for the purpose of evaluating uropathogen biofilm control by these bacteriophages. Defined phage cocktails targeting *P. aeruginosa* and *P. mirabilis* were designed based on the biofilm inhibition screens. Hydrogel-coated catheters were pretreated with one or both cocktails and challenged with approximately 1×10^3 CFU/ml of the corresponding pathogen(s). The biofilm growth on the catheter surfaces in AUM was monitored over 72 to 96 h. Phage pretreatment reduced *P. aeruginosa* biofilm counts by 4 $\log_{10}$ CFU/cm^2 ($P \leq 0.01$) and *P. mirabilis* biofilm counts by >2 $\log_{10}$ CFU/cm^2 ($P \leq 0.01$) over 48 h. The presence of *P. mirabilis* was always associated with an increase in lumen pH from 7.5 to 9.5 and with eventual blockage of the reactor lines. The results of this study suggest that pretreatment of a hydrogel urinary catheter with a phage cocktail can significantly reduce mixed-species biofilm formation by clinically relevant bacteria.

A recently reported survey of 183 acute care hospitals in 2011 found that approximately 9% of the health care-associated infections were catheter-associated urinary tract infections (CAUTIs) (1). The increases in patient morbidity, hospital stays, and costs of care for patients with CAUTIs are substantial (2, 3).

Microorganisms may colonize indwelling urinary catheters and form extensive and often multispecies biofilm (4–8). The exact role of catheter-associated biofilms in CAUTI pathogenesis is poorly understood, but there is evidence that such biofilms play an important role as stable reservoirs of uropathogenic microorganisms that are resistant to antimicrobials (9–12) and difficult to eliminate even if the catheter is removed (13–15). Large reductions in CAUTI rates can be achieved by limiting catheterized patient days and by implementing good catheter care practices (16), but there is still substantial interest in developing urinary catheters that are highly resistant to bacterial colonization by virtue of an inherent property of the material itself or by impregnation or coating of the structural material with an antimicrobial or biological agent. Proposed strategies have included surface patterning (17, 18), novel polymers (19), instillation of catheter retention balloons with bactericidal chemicals (20, 21), bacterial interference (22, 23), and catheter coatings impregnated with antimicrobial agents (24–34). Of these, only nitrofurazone-impregnated catheters and catheters with hydrophilic and/or silver alloy-impregnated coatings have reached the U.S. market; these have shown mixed results (29–33, 35). However, a recent study by Pickard et al. (36) found that neither silver alloy- nor nitrofurazone-coated catheters significantly reduced CAUTIs in a clinical study of patients with short-term catheterization, and the clinical data are currently considered insufficient to strongly recommend their use in standard practice (16).

Our group previously showed that bacteriophages can be incorporated into a hydrogel coating on Foley silicone urinary catheters and reduce biofilm formation in an *in vitro* catheter model (37, 38). Bacteriophages are viruses that specifically infect and kill their bacterial hosts. They have potential as biofilm control agents because their specificities can be tailored to target certain pathogens, they are self-replicating in the presence of their host cells and are eliminated by the body in the absence of host cells, they can be used effectively against multidrug-resistant bacteria, and multiple phages can be combined to broaden the effective range of the treatment (38–40). In the present study, our goal was to evaluate the effectiveness of a phage cocktail-treated hydrogel silicone urinary catheter in mitigating biofilm formation by a mixture of two uropathogens in an *in vitro* model. Specifically, we chose *Pseudomonas aeruginosa* and *Proteus mirabilis* as the target uropathogens. The two species were observed together in urinary catheter biofilms (5, 41). *P. aeruginosa* is one of the most frequently iso-

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TABLE 1 Sources, clinical histories, and biofilm formation data of the bacterial strains used in this study

Strain	Isolation history	Use in this study				Biofilm formation	
		Sewage enrichment	Appelmans and host range	Isolation/ purification[a]	Efficacy screening	Mean OD_{540}	SD ($n = 4$)
P. aeruginosa							
PsAer-1	Unknown	+	+	−	−	1.1683	0.2197
PsAer-2	Unknown	−	+	+	+	0.4273	0.0983
PsAer-3	Urinary catheter	−	+	−	−	0.3798	0.0854
PsAer-4	Urine culture	+	+	+	+	0.5505	0.1503
PsAer-5	Collection site unknown	−	+	−	−	0.2003	0.0822
PsAer-6	Urine, upstream of catheter	+	+	−	+	0.8795	0.0794
PsAer-7	Urinary catheter biofilm	−	+	−	−	0.2603	0.1648
PsAer-8	Urinary catheter biofilm	+	+	−	−	0.7095	0.0817
PsAer-9	Urinary catheter biofilm	+	+	+	+	1.0625	0.2018
PsAer-10	Urine, upstream of catheter	−	+	+	−	0.4078	0.0904
PsAer-11	Urinary catheter biofilm	+	+	−	+	1.3830	1.0625
PsAer-12	Urine, downstream of catheter	−	+	−	−	0.2650	0.0840
P. mirabilis							
PrMir-1	Stool culture	+	+	−	+	0.4528	0.1103
PrMir-3	Urinary tract infection	+	+	+	+	0.3690	0.1739
PrMir-5	Urine or urinary catheter	+	+	+	−	0.5795	0.3517
PrMir-6	Urine or urinary catheter	+	+	+	+	0.2883	0.0921
PrMir-7	Urine or urinary catheter	+	+	+	−	0.3698	0.3305
PrMir-8	Urine or urinary catheter	+	+	+	−	0.2943	0.0548
PrMir-9	Urine, downstream of catheter	+	+	+	−	0.2245	0.0371
PrMir-10	Urinary catheter biofilm	+	+	+	−	0.2815	0.1348
PrMir-11	Urine, upstream of catheter	+	+	+	−	0.3230	0.0241
PrMir-12	Urinary catheter biofilm	+	+	+	−	0.3373	0.1934

[a] Most strains were used at some point during the process of individual phage purification from mixed lysates, but only the bacterial strains upon which distinct plaque morphologies ultimately persisted through 3 or 4 rounds of single plaque isolation (and thus becoming the standard "isolation and propagation host" of at least one phage isolate) are indicated here.

lated species (5, 9, 41) and is associated with serious symptomatic UTIs and CAUTIs that progress to bacteremia (5, 42). When growing in biofilms, it is also known for generating an abundance of morphological variants with various treatment susceptibilities (38, 43–45). *P. mirabilis* is a less common CAUTI-associated pathogen, but it is the primary cause of mineral-encrusted catheters, which increase the risk of complications, such as pyelonephritis and bloodstream infections (46–49).

MATERIALS AND METHODS

Media, buffers, and growth conditions. Bacterial isolates were grown on Trypticase soy agar (TSA) plates or in tryptic soy broth (TSB). Soft agar overlays for the phage enumeration assays were composed of 15 g/liter gelatin, 8 g/liter agar, 5 g/liter peptone, 3 g/liter sodium chloride, 3 g/liter beef extract, and 0.5 g/liter anhydrous manganous sulfate.

The final chemical composition of artificial urine medium (AUM) was as described by Brooks and Keevil (50). All reagents except iron sulfate, calcium chloride, and lactic acid were added to a large flask and dissolved in autoclaved reverse-osmosis (RO) water to approximately 90% of the final volume; the remaining reagents were slowly added to the constantly stirring solution (iron sulfate from freshly prepared concentrated stock, and calcium chloride dissolved in some of the remaining water and added slowly to prevent precipitation); sterile water was added to reach a final volume, the pH was adjusted to 6.8, and AUM was vacuum filtered through a 0.2-μm surfactant-free cellulose acetate filter and stored at room temperature. Also critical to preventing salt precipitation during storage, all glassware was rinsed with 3 to 6 M HCl and then RO water prior to sterilization and use.

A human urine sample was collected from one healthy donor over a 24-h period and stored at 4°C during collection. The urine sample was vacuum filtered through a series of three filters, 0.45 μm, 0.2 μm, and 0.1 μm. The aliquots were stored at −20°C until just prior to use.

The bacterial suspensions generally were prepared in Butterfield buffer (BB) (42.5 mg/liter KH_2PO_4 [pH 7.2]). The phage suspensions and dilutions generally were prepared in phage storage buffer (PSB) (5.84 g/liter NaCl, 1.06 g/liter Tris-HCl, 0.39 g/liter Tris base, 2.46 g/liter $MgSO_4 \cdot 7H_2O$).

All incubations were at 37°C unless indicated otherwise, and all broth cultures were incubated on an orbital shaker at 100 rpm.

Selection of bacterial strains. Thirty-five isolates of *P. aeruginosa* and 39 isolates of *P. mirabilis* (almost exclusively clinical and urinary tract related) were taken from existing lab collections within the Clinical and Environmental Microbiology Branch at the CDC. These were screened for biofilm formation ability, as described below; 12 *P. aeruginosa* and 10 *P. mirabilis* isolates were selected for further use and are described in Table 1. Seven additional strains of *P. aeruginosa* from other sources were also used for their respective phages: EAMS2005-A, EAMS2005-B, EAMS2005-C (38), 31, 109, M4, and M6 (70).

Bacteriophage isolation, purification, propagation, and lytic spectrum. One-liter samples of untreated sewage were collected from the Snapfinger Creek wastewater treatment facility in DeKalb County, GA. *P. aeruginosa* phages were enriched from each of the four samples as follows: 50 ml of AUM in a 250-ml Erlenmeyer flask was spiked with 5×10^7 CFU each of six *P. aeruginosa* strains (see Table 1), the flasks were incubated for 2 h at 37°C on an orbital shaker at 100 rpm, 50 ml of sewage was added to each flask, and the mixture was returned to the shaker for overnight incubation. Chloroform was added to each flask (2% [vol/vol]), and the flasks were returned to the shaker for 20 min. The purpose of adding chloroform was to lyse the phage-infected bacterial cells, kill the bacterial

cells that were not infected, and isolate phages. The crude lysate was then centrifuged at 8,000 × *g* and 4°C for 20 min. The supernatant was vacuum filtered through a 0.22-μm pore surfactant-free cellulose acetate filter and stored at 4°C. The *P. mirabilis* phages were similarly enriched but using 50 ml of TSB and 5×10^7 CFU each of 10 *P. mirabilis* strains (see Table 1).

Appelmans passage was conducted on the mixed lysates in an attempt to increase the likelihood of isolating good biofilm-inhibiting phages that might have been present in too low a concentration to be detected after the initial enrichment. Appelmans passage is a long used but largely unpublished technique based on a study by Appelmans (51) that is used to expand the host range of a phage mixture. Briefly, the phage inoculum contained mixed lysate from an initial sewage enrichment; this mixture was serially diluted 10-fold in BB. Six tubes of AUM were each inoculated with 2×10^6 CFU/ml of one *P. aeruginosa* strain. Five of these tubes were then inoculated with 100 μl of a 10-fold phage dilution, one each for the undiluted phage mixture through the 10^{-4} dilution. This was done with 12 *P. aeruginosa* strains (see Table 1), and the tubes were incubated on an orbital shaker at 37°C. After approximately 18 h, each tube was checked for lysis. All tubes showing visible evidence of at least partial lysis, regardless of host strain, were combined into one mixed lysate, termed Appelmans 1. The Appelmans 1 lysate was centrifuged, filtered (0.22 μm), and diluted for use in a second round. Passage was repeated 3 times, and the filtered Appelmans 4 lysate was retained. The procedure was repeated for *P. mirabilis* phages using 25% TSB. The individual phage isolates were then purified separately from each of the original mixed lysates and from Appelmans 4. Each of these was diluted 10-fold in BB and plated on each of seven *P. aeruginosa* cultures or nine *P. mirabilis* strain cultures, as appropriate (see Table 1). Unique plaque morphologies were purified by at least three rounds of single plaque isolation. The resulting phage isolates are shown in Tables S1 and S2 in the supplemental material. Individual phages were sized using pulsed-field gel electrophoresis (PFGE), using the following conditions: briefly, phage stock was mixed 1:1 with 1.4% PFGE agarose to create plugs. The phage capsids were lysed in the plug, using 0.5 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) in 1% *N*-lauryl sarcosine (Sigma-Aldrich) with 0.2% SDS at 55°C. The plugs were washed 4 times in Tris-EDTA (TE) buffer (Fisher Scientific, Pittsburgh, PA) and sealed into a 1% PFGE agarose gel. The gels were run for 18 h at 6 V/cm, with a 2-s initial switch time and a 10-s final switch time. The gels were stained with ethidium bromide, and the genomic DNA bands were compared to an XbaI-digested *Salmonella enterica* serovar Braenderup standard to estimate their sizes.

Phage stocks were propagated in liquid culture in AUM (*P. aeruginosa* phages) or 25% TSB (*P. mirabilis* phages), using the host on which they were originally isolated and purified (52). The phage lytic spectrum was determined using the spot plate assay (38).

Biofilm formation assays and method for screening phage for biofilm inhibition. Biofilm formation was quantified using a crystal violet (CV) assay modified from O'Toole and Kolter (53). This assay was used to assess the biofilm formation of the untreated and phage-treated bacterial isolates. For each bacterial isolate being tested, 10 μl of a 16-h culture in TSB was used to inoculate 190 μl of AUM in a 96-well flat-bottom tissue culture plate. A fixed volume of 16-h liquid culture was used to inoculate each well of the CV assay, since standardizing the initial concentrations of 80 isolates at a time was not practical. However, dilution plating of randomly selected 16-h cultures showed no noticeable viable count differences among isolates of the same species. The plates were incubated at 35°C on a rocker shaker for 10 h. Fifty microliters of 1% Turks stain (10 g/liter CV, 3% [vol/vol] glacial acetic acid) was added to each well. After 15 min at room temperature (no shaking), all liquid was carefully aspirated from each well. Excess CV was rinsed away by three rounds of careful addition and aspiration of 250 μl of sterile distilled water to each well. Biofilm-associated CV was eluted into the well by adding 350 μl of 95% ethanol to each well, which was pipetted up and down three times. Two hundred microliters per well was transferred to a new 96-well plate, and optical density at 540 nm (OD_{540}) readings were collected using a BioTek Synergy 2 plate reader (BioTek Instruments, Winooski, VT). Four independent replicates were run in each experiment.

To screen the phage collection for biofilm control ability, microtiter plates containing 1×10^6 CFU/ml of a log-phase bacterial culture in AUM and 5×10^5 PFU/ml of the test phage were incubated for 18 h. Each of the bacterial strains was tested against each individual phage for that species with medium-only negative-control wells and phage-free positive-control wells. Table 1 indicates the bacterial strains that were used for this efficacy screening both alone and in strain mixtures.

AUM validation. In order to validate the use of AUM as a urine analog for phage efficacy screening, a subset of the single-phage and phage cocktail efficacy screens were run side-by-side in AUM and human urine. Biofilm formation by *P. aeruginosa* strain PsAer-9 was tested in the presence of each individual phage, and biofilm formation by a mixture of *P. aeruginosa* strains PsAer-2, PsAer-4, PsAer-9, and PsAer-11 was tested in the presence of phage cocktails. Biofilm formation in AUM was assessed using the CV staining protocol described above, except that anhydrous ethyl alcohol was used to elute the biofilm-bound CV. Biofilm formation in human urine was assessed using a metabolic activity assay adapted from Smith and Hunter (54). Following the 16-h biofilm growth incubation period, all liquid, including suspended cells, was carefully aspirated from each well, and the wells were rinsed once by adding and aspirating phosphate-buffered saline. Two hundred fifty microliters of 0.5 mg/ml XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] (Molecular Probes, Carlsbad, CA) and 50 μM menadione solution, prepared as described by Smith and Hunter (54), was added to each well under low-light conditions. The plates were incubated at room temperature in the dark for 2 h. The soluble well contents were mixed by pipetting, 200 μl per well was transferred to a new 96-well plate, and OD_{492} readings were collected using the plate reader, as described above.

Catheter reactors. The biofilm prevention efficacy of the final phage cocktails was determined in a flowing catheter reactor model over a three- to four-day period. The *in vitro* model system for growing biofilms on hydrogel-coated silicone Foley catheters (16 French [Fr.] Lubri-Sil; C. R. Bard, Covington, GA) was based on the modified drip flow reactors (mDFRs) described previously (38). Four lengths of catheter (approximately 8 in. each) were held in a custom-made square plastic tray, and each catheter was separately connected to sterile medium, bacterial inoculum, and waste flasks. The feed lines from the sterile medium flask were fitted with flow breaks to prevent upstream migration of bacteria and contamination of the sterile feed flask. The catheter assembly was sterilized with ethylene oxide. The flask and tubing assemblies were autoclaved, and the complete system was assembled aseptically. Prewarmed AUM was added to the sterile medium flask (6 liters) and the bacterial inoculum flask (0.5 liters).

Three sets of experiments were conducted: (i) anti-*Pseudomonas* phage cocktail was challenged with *P. aeruginosa* PsAer-9, (ii) anti-*Proteus* phage cocktail was challenged with *P. mirabilis* strain PrMir-12, and (iii) the two phage cocktails together were challenged with a mixture of both bacteria. The anti-*Pseudomonas* phage cocktail contained 6 phages, ΦPaer4, ΦPaer14, M4, 109, ΦE2005-A, and ΦE2005-C, at 1×10^9 PFU/ml each. The anti-*Proteus* phage cocktail contained 4 phages, ΦPmir1, ΦPmir32, ΦPmir34, and ΦPmir37, at 3×10^8 PFU/ml each. The cocktails were prepared from individual phage stocks that had been passed through a 0.22-μm filter and applied to sterile 300-kDa Macrosep diafiltration columns to replace the culture medium with phage buffer. The bacterial strains used for challenge were grown overnight in AUM and subcultured a few hours before the start of the experiment. Immediately prior to the bacterial inoculum step, the cultures were sonicated in a water bath and vortexed to evenly disperse the cells, and a 0.5 McFarland standard cell suspension was prepared in prewarmed AUM.

The experimental timeline for each reactor was as follows: (i) each catheter segment was filled with the phage cocktail or phage-free control buffer (delivered via injection port immediately upstream of the catheter) and incubated for 1 h, (ii) bacteria were added to the stirring inoculum

flask to achieve an initial concentration of approximately 1×10^3 CFU/ml, (iii) bacterial inoculum was pumped through the catheters at 1 ml/min for 2 h, and (iii) the bacterial flow was stopped, and sterile medium was pumped through the catheters at 0.5 ml/min for up to 4 days. The experiments were conducted at 35°C.

Recovery and enumeration of biofilm organisms and phages. Inoculum flask samples were collected at the start and end of the 2-h bacterial inoculation period. The catheter samples were collected 2 h, 24 h, 48 h, and 72 h (96 h for *P. aeruginosa* reactors) after the initiation of bacterial inoculation. The catheters were aseptically removed from the holding trays, and the outer surface was disinfected. The lumen fluid was drained and stored on ice. Four 1-cm sections were cut from the center of the catheter. Each section was sliced in half longitudinally, rinsed gently in PSB, and both halves were placed in a 16- by 100-mm screw-cap glass tube containing 3 ml of cold PSB and placed on ice. The lumen and catheter sample tubes were processed as previously described: the tubes were placed in a water bath sonicator (42-kHz Branson 2510; Branson, Danbury, CT) for 10 min, vortexed for 30 s, sonicated for 5 min, vortexed for 30 s, sonicated for 30 s, vortexed for 30 s, and returned to ice.

The lumen samples were serially diluted and plated as spread plates for bacterial quantification and on soft agar overlay plates for phage detection. At the 2-h time point, the cell suspensions from the catheter pieces and lumen samples were concentrated by vacuum filtration onto 0.22-μm gridded nitrocellulose filter membranes and the membranes transferred to agar plates. At all other time points, the cell suspensions recovered from the catheter pieces were serially diluted and plated for bacterial quantification. *Pseudomonas* isolation agar (PIA) (BD Difco, Franklin Lakes, NJ) was used for the *P. aeruginosa* experiments, CI_{50} agar (modified from Clayton, Chawla, and Stickler [14] to contain 50 mg/liter colistin) was used for the *P. mirabilis* experiments, and all samples were plated on both media for the two-species experiments. The one exception to this was for the 2-h time point in the two-species experiments, in which only one filter membrane was prepared per sample and placed on MacConkey II agar (BD Difco). MacConkey II allowed visual differentiation of *P. aeruginosa* and *P. mirabilis* when they were present in small and approximately equal numbers, as was expected at this time point. Lumen pH was measured using pH test strips (Ricca Chemical, Arlington, TX).

Statistical analyses. All OD_{540} data from the 96-well plate biofilm assays were standardized by subtracting the absorbance of the cell-free blank well from the absorbance of each subject well within that replicate. For the phage efficacy data, the standardized absorbance for each sample well was then expressed as a percentage of the standardized absorbance for the phage-free control well. The data were analyzed using the generalized linear model procedure in the Statistical Analysis System (SAS) version 9.2 software (SAS Institute), with a Dunnett's test for multiple comparisons to the untreated (phage-free) control. For the catheter reactor experiments, bacterial and phage concentrations (or CFU/cm^2 for the biofilm bacteria recovered from catheters) were $\log_{10}$ transformed prior to analysis. For the biofilm bacterial counts, the median value of the 4 subsamples per catheter was taken as the estimate of the biofilm population size for that experimental replicate. The data were analyzed separately for each time point. The model included the number of bacterial species present, the presence or absence of phage pretreatment, and an interaction between these two factors.

RESULTS

Selection of bacterial strains. Twelve *P. aeruginosa* isolates and 10 *P. mirabilis* isolates (Table 1) were chosen as test strains for use during subsequent phage enrichment and isolation based on the following criteria: (i) good biofilm-forming ability (high normalized mean OD_{540} in the crystal violet assay), (ii) the repeatability of biofilm formation (low standard deviation [SD] of normalized OD_{540}), (iii) the strains were derived from different patients and studies, and (iv) collectively, the strains encompassed several different, though individually stable, colony morphologies.

Phage isolation and characterization. The phages were enriched from untreated sewage samples by spiking the sewage samples with equal amounts of ≥6 strains of the desired host species. The mixed enrichment lysates were then plated on multiple host strains to facilitate the detection of as many phages as possible. After ≥3 rounds of single plaque purification in which a consistent plaque morphology was observed, our collection included 34 new *P. mirabilis* phage isolates, 27 new *P. aeruginosa* phage isolates, and 9 *P. aeruginosa* phages from other studies. Some of the new isolates were likely multiple isolations of the same phage from different host strains. We therefore tested the host range of each isolate using the spot plate assay and estimated each phage genome size by PFGE (see Tables S1 and S2 in the supplemental material). Most of the *P. aeruginosa* phages infected at least half of the clinical *P. aeruginosa* strains tested (see Table S1). Notable exceptions included the *P. aeruginosa* strains PsAer-3 and PsAer-5, which were strongly lysed by very few of the phages in our collection. *P. mirabilis* strains PrMir-5, PrMir-6, PrMir-7, PrMir-8, PrMir-9, PrMir-10, PrMir-11, and PrMir-12 were all infected by most *P. mirabilis* phages (see Table S2). Several *P. mirabilis* and *P. aeruginosa* phages produced a visible but extremely faint dimpling effect on certain bacterial lawns, possibly indicating enzymatic activity rather than productive infection.

Validation of AUM and phage efficacy screening. A defined urine analog was used for this study because the large amounts of urine required for the catheter reactor experiments (>6 liters each) made it impractical to use filtered human urine. In order to determine whether the conclusions drawn from the AUM-based experiments would be similar to the results using human urine, a subset of the phage efficacy screens was run simultaneously in AUM and filtered human urine. Biofilm formation in AUM was assessed with CV, which stains all adherent biomass attached to the well surfaces, whereas biofilm formation in human urine was assessed with XTT, which is based on the ability of viable cells in surface-adherent biofilm to reduce the tetrazolium salt XTT to a water-soluble orange-colored product. Neither assay could be used for both AUM and human urine. In general, human urine supported more abundant biofilm growth than AUM. The XTT assay was not sensitive enough to use with AUM-grown bacteria, and no tested acetone-ethanol mixtures completely destained the thickest CV-stained biofilms grown in human urine, which prevented differentiation among treatment groups even when large differences were obvious to the naked eye.

Despite the different underlying biological concepts of the CV and XTT assays, the results of the AUM and human urine experiments were almost identical. Of 35 individual phages tested against *P. aeruginosa* PsAer-9, the classification of each as effective (significantly less bacterial accumulation or activity than that in the phage-free control, $P < 0.05$) or ineffective (no difference from that of the phage-free control, $P > 0.05$) differed for only 3 phages: ΦPaer27 was deemed ineffective in AUM but not in human urine, and ΦPaer16 and ΦE2005-C were deemed ineffective in human urine but not in AUM (data not shown).

The AUM-CV assay was used to determine the efficacies of all of the *P. aeruginosa* and *P. mirabilis* phages in our collection against the biofilms of several strains of their target species. Figure 1 shows the results of *P. aeruginosa* phage efficacy screening against biofilms of *P. aeruginosa*. Three different *P. aeruginosa* strains were chosen for screening (*P. aeruginosa* PsAer-2, PsAer-4, and PsAer-9) to reduce bias when selecting phages for down-

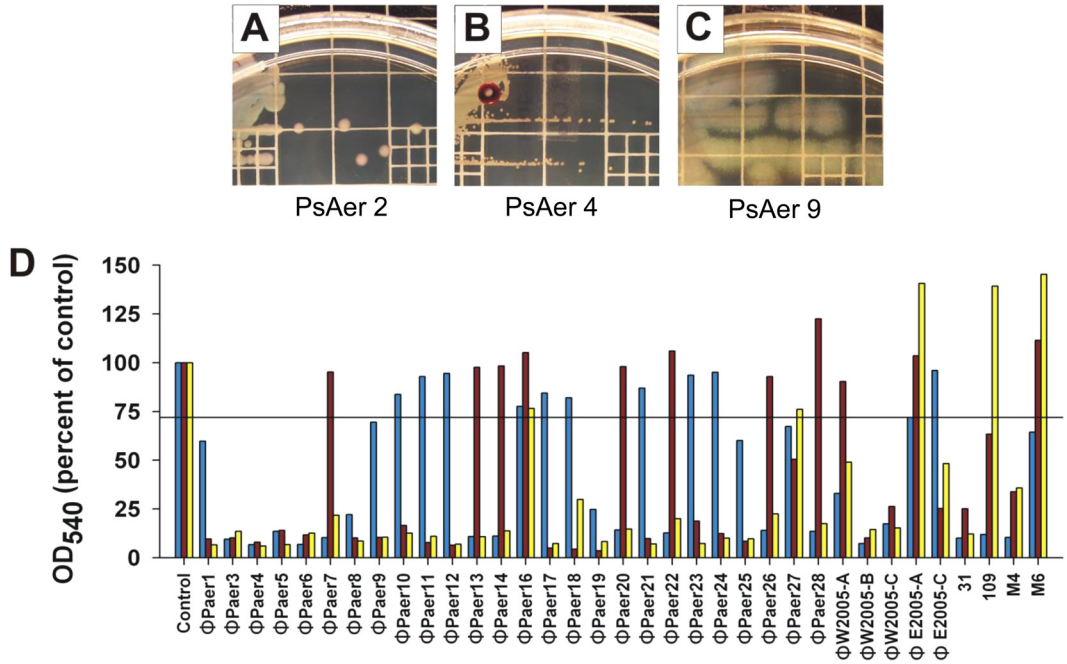

FIG 1 Efficacy screening of *P. aeruginosa* phages. (A to C) *P. aeruginosa* isolates selected for phage efficacy screening, showing colony morphologies on PIA plates. (D) Total biofilm mass accumulation by individual *P. aeruginosa* strains (blue bars, PsAer-2; red bars, PsAer-4; yellow bars, PsAer-9) following coinoculation with a single phage. The initial concentrations of bacteria and phage in each well were 1×10^7 CFU/ml and 5×10^5 PFU/ml, respectively. The mean OD_{540} readings below the horizontal cutoff line indicate a significant ($P < 0.05$, Dunnett's test) reduction in biofilm mass by that phage-host combination.

stream catheter reactor experiments. The colony morphologies of these chosen bacterial strains (Fig. 1) were stable, and the *P. aeruginosa* phages exhibited different host range patterns on each isolate. Eleven of the phages tested significantly reduced the amount of biofilm formed by all three *P. aeruginosa* strains. There was no obvious correlation between colony morphology and phage susceptibility in this assay. Phage efficacy against two other *P. aeruginosa* strains (PsAer-6 and PsAer-11) was also determined. Biofilm formation by *P. aeruginosa* PsAer-6 was unaffected by any phage under these conditions, even those that produced clearing in the spot plate assays (data not shown). In an effort to find any phages that could affect biofilm development by this strain, the highest available concentrations of each phage were tested; M4 and ΦE2005-A were able to reduce biofilm formation by *P. aeruginosa* PsAer-6 to 12% and 31% of that of the control, respectively (both $P < 0.001$), when initially present at approximately 1×10^9 PFU/ml. Phages ΦE2005-C and 109 were effective against *P. aeruginosa* PsAer-11 biofilms using the higher phage titer (1×10^9 PFU/ml) (data not shown). Insufficient biofilm development of the 6 tested *P. mirabilis* strains in the AUM-CV assay prevented an evaluation of phage efficacy against *P. mirabilis* biofilms.

Selection of bacterial isolates and phage cocktail for catheter reactors. Based on the results of the phage screening assays, the phage cocktail used in the catheter model studies contained the following *P. aeruginosa* phages: ΦPaer4, ΦPaer14, M4, 109, ΦE2005-A, and ΦE2005-C. *P. mirabilis* phage selection for the catheter reactor experiments was based on host range, as determined by the spot plate assay and genome size diversity, as well as the ease of producing high-titer lysates. *P. aeruginosa* PsAer-9 was selected for the catheter model based upon its broad susceptibility to individual phages (Fig. 1). *P. mirabilis* PrMir-12 was selected because it exhibited broad susceptibility to *P. mirabilis* phages (see Table S2 in the supplemental material). Both organisms were urinary catheter biofilm isolates (Table 1).

Selective quantification of bacteria and phages recovered from catheter reactors. Because of the need to differentiate between *P. aeruginosa* and *P. mirabilis* recovered from mixed-culture catheter biofilms, two selective media were used: *Pseudomonas* isolation agar (PIA) for *P. aeruginosa* and CI_{50} agar for *P. mirabilis*. Each medium was selective for its respective target (≥100,000-fold reduction in the apparent viable count of the nontarget species) and gave the same viable counts for that target species as did nonselective TSA. Species selectivity was maintained even when high initial concentrations of nontarget bacteria were seeded in TSA-based soft agar overlays. As a result, when samples from mixed-culture catheter reactors were plated in the overlays, we reliably detected phages of one bacterial species at a time simply by seeding the lawn with the appropriate host species. It is important to note that CHROMagar Orientation (BD, Sparks, MD) did not give reliable differentiation when colonies of the two species grew near each other, and *M*-PA-C agar (BD, Sparks, MD) did not sufficiently inhibit *P. mirabilis* grown in AUM. In addition, colistin tolerance in *P. mirabilis* varied widely among the strains.

Bacterial and phage populations in catheter reactors over time. The anti-*Pseudomonas* cocktail contained six *P. aeruginosa* phages at 1×10^9 PFU/ml each, chosen based on the results of the screening assays. Phage 109 appeared to substantially improve biofilm reduction in the cocktail screens; in the individual screens, only phages 109 and ΦE2005-C and M4 and ΦE2005-A significantly reduced biofilm formation by *P. aeruginosa* PsAer-11 and PsAer-6, respectively. Phages ΦPaer4 and ΦPaer14 both reduced

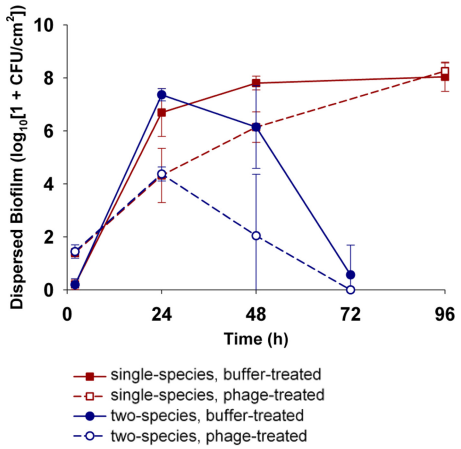

FIG 2 Effect of phage pretreatment of silicone hydrogel catheters on biofilm formation by *P. aeruginosa*. Closed squares, $\log_{10}$ mean biofilm *P. aeruginosa*, single-species reactor, untreated; open squares, $\log_{10}$ mean biofilm *P. aeruginosa*, single-species reactor, phage treated; closed circles, $\log_{10}$ mean biofilm *P. aeruginosa*, two-species reactor, untreated; open circles, $\log_{10}$ mean biofilm *P. aeruginosa*, two-species reactor, phage treated. The error bars represent the standard deviation, $n = 3$ or 4.

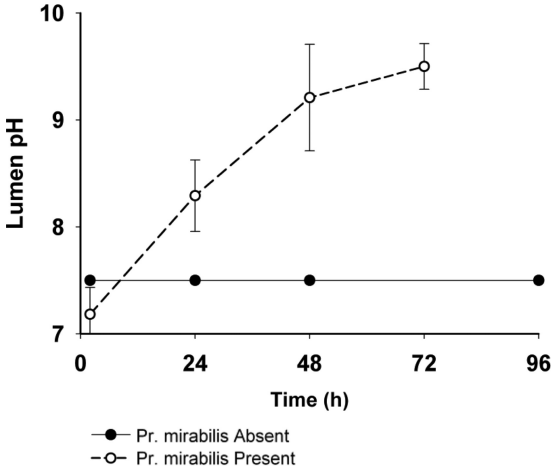

FIG 4 pH of the catheter luminal fluid, pooled across experiments in the presence (open circles) and absence (closed circles) of *P. mirabilis* in the catheter reactor system. The error bars represent the standard deviation, $n = 3$ or 4.

biofilm formation by all other strains in individual phage screens but with slightly different host ranges. Together, the six phages in this cocktail (i.e., 109, ΦE2005-C, M4, ΦE2005-A, ΦPaer4, and ΦPaer140) infected all 12 *P. aeruginosa* spp. and 2 *Pseudomonas* spp. used in the spot tests. The anti-*Proteus* phage cocktail contained four *P. mirabilis* phages at 3×10^8 PFU/ml each. Together, the four phages in this cocktail (i.e., ΦPmir32, ΦPmir34, ΦPmir1, and ΦPmir37) infected all 10 *P. mirabilis* strains used in the spot tests.

Figure 2 shows the effect of phage pretreatment of catheters on biofilm formation by *P. aeruginosa* without phage pretreatment. The biofilm *P. aeruginosa* levels in the single-species reactor were 6.69 log CFU/cm^2 at 24 h and increased to 7.80 log CFU/cm^2 at 48 h; these levels were essentially maintained through 96 h. Coinoculation with *P. mirabilis* resulted in higher *P. aeruginosa* counts at 24 h (7.36 log CFU/cm^2) but substantially lower counts after 48 h (6.14 log CFU/cm^2). *P. aeruginosa* biofilms were not detected at 72 h, suggesting inhibition associated with the presence of *P. mirabilis*. The results of an experiment in which *P. aeruginosa* was grown in planktonic culture in the presence of *P. mirabilis* (Fig. 3) suggested that *P. mirabilis* did not directly inhibit the growth of *P. aeruginosa* in the catheter model system. However, a substantial increase in luminal pH levels in catheter reactors containing *P. mirabilis* (Fig. 4) and a marked reduction in the *P. aeruginosa* PsAer-9 counts over time when the organism was grown at elevated pH in 25% TSB (Fig. 5) suggested that the inhibition of *P. aeruginosa* observed in the two-species reactors was associated with elevated pH.

In single-species catheter reactors containing *P. aeruginosa* only, phage cocktail pretreatment reduced biofilm levels by approximately 2.5 log after 24 h (4.31 ± 1.02 versus 6.69 ± 0.91 $\log_{10}$ CFU/cm^2; $P \leq 0.01$) (Fig. 2). The biofilm levels partially rebounded on phage-pretreated catheters at 48 h (7.80 ± 0.26 versus 6.14 ± 0.57 $\log_{10}$ CFU/cm^2), but the 1.5-log reduction was significant ($P \leq 0.01$). In the two-species experiments, the *P. aeruginosa* populations on phage-treated catheters were smaller than those on untreated catheters ($P \leq 0.01$), approximately 3 orders of mag-

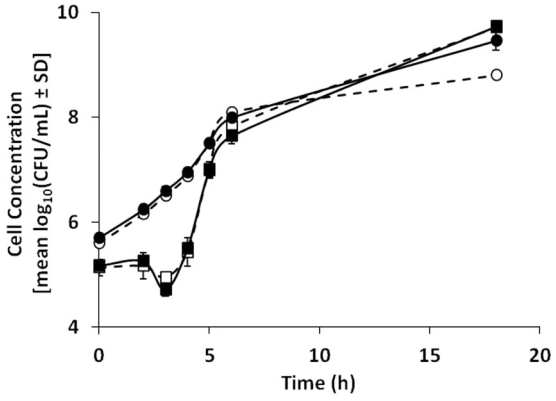

FIG 3 Mean viable cell count ($\log_{10}$ CFU/ml) of *P. aeruginosa* PsAer-9 alone (closed squares) and in coculture with *P. mirabilis* PrMir-12 (open squares), and *P. mirabilis* PrMir-12 alone (closed circles) and in coculture with *P. aeruginosa* PsAer-9 (open circles), in 25% tryptic soy broth, over 18 h. The error bars represent the standard deviation.

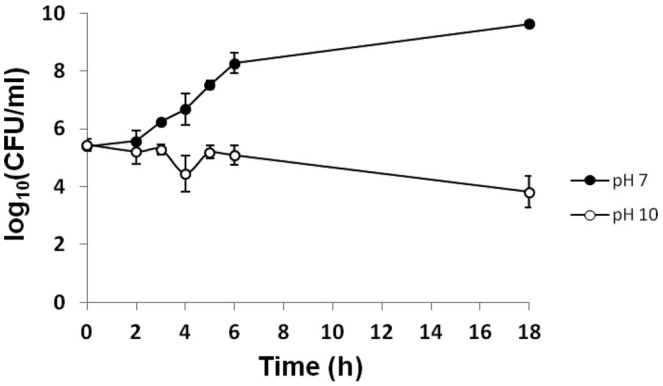

FIG 5 Mean viable $\log_{10}$ cell count (CFU/ml) of *P. aeruginosa* when grown in 25% tryptic soy broth adjusted to pH 7 or pH 10 for 18 h. The error bars represent the standard deviation.

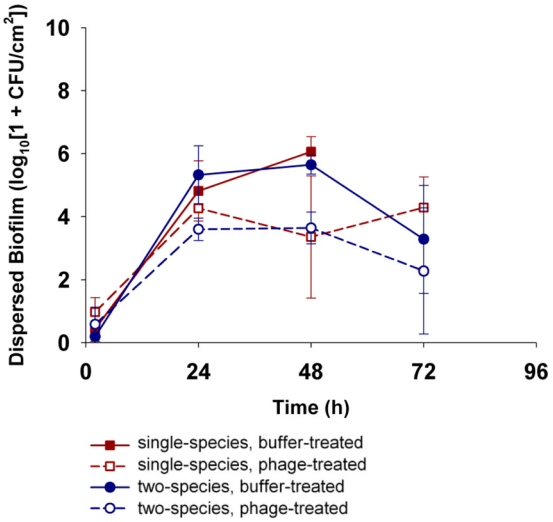

FIG 6 Effect of phage pretreatment of silicone hydrogel catheters on biofilm formation by *P. mirabilis*. Closed squares, log_{10} mean biofilm *P. mirabilis*, single-species reactor, untreated; open squares, log_{10} mean biofilm *P. mirabilis*, single-species reactor, phage treated; closed circles, log_{10} mean biofilm *P. mirabilis*, two-species reactor, untreated; open circles, log_{10} mean biofilm *P. mirabilis*, two-species reactor, phage treated. The error bars represent the standard deviation, $n = 3$ or 4.

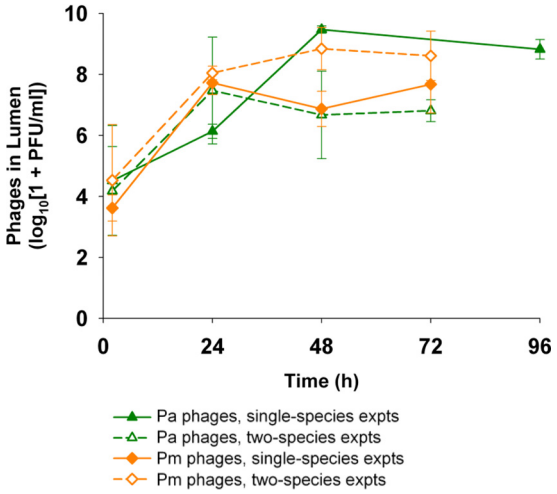

FIG 7 Mean number of phage (log_{10} [1 + PFU/ml]) in the luminal fluid of catheters in the catheter reactor system. Closed triangles, total *P. aeruginosa* (Pa) phages, single-species experiment; open triangles, total *P. aeruginosa* phages, two-species experiment; closed diamonds, total *P. mirabilis* (Pm) phages, single-species experiment; open diamonds, total *P. mirabilis* phages, two-species experiment. The error bars represent the standard deviation, $n = 3$ or 4.

nitude at 24 h (4.37 ± 0.26 versus 7.36 ± 0.23 log_{10} CFU/cm^2) and 4 orders of magnitude at 48 h (2.05 ± 2.31 versus 6.14 ± 1.56 log_{10} CFU/cm^2). However, the *P. aeruginosa* population was eliminated by 72 h, regardless of whether phages were present. We did not observe frequent colony morphology variants among the biofilm *P. aeruginosa* recovered from the catheter surfaces. However, when randomly selected colonies were transferred from PIA to TSA, brick-red colonies and small-colony variant (SCV) morphologies were sometimes observed, suggesting that such variants were present among the recovered adherent cells but were simply not apparent on PIA medium. Figure 6 shows the effect of phage pretreatment of catheters on biofilm *P. mirabilis*. In single-species catheter reactors with *P. mirabilis*, biofilm development at 24 h was not significantly different in the catheters that were pretreated with the phage cocktail compared to that in the untreated catheters (4.26 ± 0.07 versus 4.81 ± 0.96 log_{10} CFU/cm^2, respectively; $P = 0.38$). At 48 h, however, approximately 2.5 orders of magnitude fewer bacterial cells were recovered from the phage-treated catheters compared to that in the untreated catheters (3.35 ± 1.94 versus 6.06 ± 0.48 log_{10} CFU/cm^2, respectively). In the two-species experiments, *P. mirabilis* populations were approximately 2 orders of magnitude smaller on the phage-treated catheters than those on the untreated catheters at both 24 h (3.59 ± 0.36 versus 5.33 ± 0.92 log_{10} CFU/cm^2, respectively; $P < 0.05$) and 48 h (3.64 ± 0.50 versus 5.64 ± 0.30 log_{10} CFU/cm^2, respectively; $P < 0.01$). At 72 h, the *P. mirabilis* populations on both the treated and untreated catheters were declining, although not as drastically as were the *P. aeruginosa* populations in the same catheters. Most reactors containing *P. mirabilis* became fully blocked between 54 and 72 h, apparently due to the occlusion of the narrower silicone tubing upstream or downstream of the catheter, rather than being caused by occlusion of the catheter itself. For this reason, the experiments with *P. mirabilis* were terminated at 72 h. It is also noteworthy that the silicone tubing downstream of the phage-treated catheters did not become occluded.

The phage populations in the lumen fluid were monitored throughout the catheter experiments (Fig. 7). The data are presented as the total number of all *P. aeruginosa* or *P. mirabilis* phages, since individual phages within the cocktails were not all reliably differentiated by plaque morphology. However, several important pieces of information can be gleaned from the aggregate phage data. First, in all experiments, at least one phage infecting each species was actively replicating on the bacteria present in the biofilm and/or the lumen fluid, with phage populations increasing from an initial lumen concentration of approximately 10^4 PFU/ml in all experiments to between 10^6 PFU/ml (*P. aeruginosa* phages at 24 h in the single-species experiments) and 10^9 PFU/ml (*P. aeruginosa* phages at 48 h in the single-species experiments). Among the recovered adherent cells, extensive phage activity was observed, both as phage nibbling around colony edges on plates with isolated colonies and as plaque formation on the plates with confluent bacteria growth. Second, this replication occurred at a high pH, with lumen phage populations for both species remaining at $>10^6$ PFU/ml, even at pH 9.5. This is in marked contrast to our experiences in batch cultures, in which the presence of *P. mirabilis* caused the pH of AUM to reach 9.5 within a few hours, and we could not amplify the phage populations. Third, at 48 h, the *P. aeruginosa* phage population was generally, though not quite significantly ($P = 0.076$), smaller in the two-species experiment, when the host population was rapidly declining. The bacterial populations in the lumen followed similar trends as the populations of the adherent cells recovered from the catheter surfaces (data not shown).

DISCUSSION

We previously investigated phages as potential antimicrobial agents for reducing biofilm development by *Staphylococcus epidermidis* (37) and *P. aeruginosa* (38). That work demonstrated that

active phages can be incorporated into a hydrogel coating on catheters and reduce biofilm formation *in vitro* and that phage cocktails can reduce biofilm formation for longer time periods than single phages can. Our current study expanded upon that work by studying these phage-biofilm interactions against a two-species bacterial biofilm using a validated urine analog as the growth medium, low initial bacterial inocula, 3- to 4-day experimental timelines, and phage cocktails against both bacterial species. We chose *P. aeruginosa* and *P. mirabilis* as target uropathogens. These two species are observed together in urinary catheter biofilms (5, 41). *P. aeruginosa* is one of the most frequently isolated species (5, 9, 41) and is associated with serious symptomatic UTIs and CAUTIs that progress to bacteremia (5, 42). When grown in biofilms, this organism is also known for generating an abundance of morphological variants with various treatment susceptibilities (38, 43–45). *P. mirabilis* is a less common CAUTI-associated pathogen, but it is the primary cause of mineral-encrusted catheters, which increase the risk of complications, such as pyelonephritis and bloodstream infections (46–49).

The AUM described by Brooks and Keevil (50) was chosen for this work because of the systematic manner in which it was formulated, our observations that CAUTI-associated isolates of *P. mirabilis* growing in AUM caused a pH increase and mineral precipitation similar to that observed in human urine, the explicit inclusion of trace amounts of iron in the medium, the absence of whole protein, and the inclusion of the smallest amounts of undefined "rich" components, such as peptone or yeast extract compared to that with other published formulations (55–58). In addition, there is evidence from other work that it supports the expression of relevant biofilm phenotypes. When Jones et al. (59) compared the structures of *P. mirabilis* biofilms grown in Brooks and Keevil AUM versus LB Miller broth, they found significant differences in surface coverage, biofilm thickness, water channel conformation, and the incidence of the swarmer cell morphology that is characteristic of *P. mirabilis*. Because our catheter model required the use of up to 20 liters of urine for a single experiment, and because human urine was difficult to filter sterilize, we chose to use the AUM formulation of Brooks and Keevil (50). The suitability of this AUM formulation as a urine analog was confirmed by the results of phage efficacy screening, in which essentially the same phages were identified as significantly reducing biofilm formation in AUM and human urine.

The most important aspect of this study was the use of a two-species biofilm model. We are aware of only three previous studies involving phage interactions with two-species biofilms, all of which involved phage treatment of preformed biofilms separate from any specific clinical situation (60–62). In our phage-free reactors, the total number of biofilm-associated *P. mirabilis* cells appears to have been smaller than the number of biofilm-associated *P. aeruginosa* in both the one- and two-species reactors. This is consistent with our biofilm and phage screens in 96-well plates, as well as with a previous report that *P. mirabilis* biofilm populations were smaller than *P. aeruginosa* biofilm populations when each was grown in the same bladder model system (20). Some studies have reported synergistic increases in biofilm mass in mixed- versus single-species biofilms (63, 64), but only Macleod and Stickler (41) specifically examined a coculture of *P. aeruginosa* and *P. mirabilis* in catheter biofilms, and they found minimal antagonism between the two species. Similarly, in our phage-free reactors, the numbers of biofilm-associated *P. aeruginosa* and *P. mirabilis* were not affected by the presence of the other species at the 2-h, 24-h, and 48-h time points. The elimination of *P. aeruginosa* by 72 h in the two-species catheter reactors, regardless of phage treatment, was likely driven by the high pH that developed between 48 and 72 h due to *P. mirabilis* urease activity, as evidenced by *P. aeruginosa* growth inhibition in medium with pH 10. However, the high pH did not appear to inhibit lytic activities for either the *P. mirabilis* or *P. aeruginosa* phages in our catheter model, suggesting that phage application in indwelling urinary catheters with high pH conditions might be feasible.

Although the application of anti-*P. aeruginosa* and anti-*P. mirabilis* phages to catheters will be ineffective against other bacterial species that may colonize the catheter, both organisms evaluated in this study play an important role in CAUTIs (5, 42) and other complications related to the use of urinary catheters (46–49).

The timeline of phage efficacy is also important for clinical utility. Previous studies with phage-treated catheters (38) have demonstrated that phage may delay but not prevent biofilm formation, resulting in a rebound effect with prolonged exposure. We also observed a rebound for both *P. aeruginosa* and *P. mirabilis*. However, the extensive phage nibbling and plaque formation observed on the plates with recovered adherent bacteria imply that large fractions of the surviving bacterial population were not truly phage resistant. Alternative explanations are that the surviving biofilm cells were transiently nonsusceptible to phage infection due to metabolic changes (especially cells deeper in the biofilm), did not support phage replication following infection (pseudolysogeny, implied in Kay et al. [62]), or existed in "spatial refuges" in which nonsusceptible cells physically shield susceptible cells from phage attack (60, 65). All of these mechanisms would support a fairly stable long-term coexistence of phage and biofilm cells, even though initial biofilm formation may be slowed. We suggest that phage pretreatment of hydrogel silicone catheters might mitigate colonization and biofilm formation by multiple organisms for short-term exposures. The observation that phages against different bacterial species when used together do not interfere with the lytic abilities of the other phages in the cocktail suggests the possibility of using combinations of cocktails to target multiple species in multispecies biofilms. The clinical relevance of this approach, with respect to the potential savings in health care costs, the reduction in costs of antimicrobials or adverse events associated with antimicrobial use, or the prevention of antimicrobial resistance by biofilm mitigation for 48 to 72 h is not clear but merits further investigation.

It may be fruitful to explore the potential synergistic interactions between phage cocktails and other antimicrobial strategies, such as catheters with more conventional antimicrobial substances, biofilm disruptors, or bacterial interference. For example, in studies of bacterial interference, a naturally nonpathogenic *Escherichia coli* strain later rendered incapable of producing P fimbriae (66) was shown to reduce colonization by *Enterococcus faecalis*, *Candida albicans*, *Providencia stuartii*, and pathogenic *E. coli* (67, 68). However, in human trials, both *P. aeruginosa* and *P. mirabilis* presented difficulties, with *P. aeruginosa* tending to overgrow the benign *E. coli* biofilm, and with the prior presence of *P. mirabilis* being predictive of poor *E. coli* persistence (22, 23, 69). A recent *in vitro* study showed that pretreating uncoated silicone catheters with both *P. aeruginosa* phages and the nonpathogenic *E. coli* strain had a synergistic effect, reducing and in some cases completely preventing *P. aeruginosa* biofilm growth on catheters

for up to 72 h, when neither phages nor bacterial interference alone was effective (45). This suggests that the combination of bacterial interference with both anti-*P. aeruginosa* and anti-*P. mirabilis* phage cocktails conceivably offers broad protection against uropathogen colonization without requiring phages that are targeted to every uropathogen of concern. It also indicates that the phage-coated catheter principle should be applicable to materials other than the hydrogel-coated catheters used in our current and previous related studies.

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The use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the U.S. CDC.

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